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13. ABSTRACT ***(Maximum 200 Words)***

There are two known receptors for estrogens, ERα and ERβ. The existence of ERβ was only recently appreciated, and little is understood about its ability to be activated by intracellular signaling pathways in the absence of estrogens. The purpose of this research program is to characterize the ability of ERβ to be activated by various ligand-independent signaling pathways, and to characterize the structural regions of ERβ, in comparison to ERα, that regulate how this receptor isotype responds to intracellular cross-talk. We have found that the originally published amino acid sequence of ERβ was incomplete, and have characterized the transcriptional activity and relative expression of the full-length and truncated forms of ERβ. In addition, the transcriptional activity of the amino-terminal, activation function of ERα is stronger than the corresponding region of ERβ. Both ERα and the short and long forms of ERβ can be activated by cAMP signaling pathways in the absence of exogenous estrogens, although the antiestrogen, 4-hydroxytamoxifen, only inhibits the activity of ERα, but not ERβ, stimulated in this manner. Taken together, ERα and ERβ are not functionally equivalent, although they do share many features, and differences between the two receptors will be exploited to understand the molecular mechanisms by which they activate transcription in the absence of estrogens.

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Introduction

Breast cancer remains the major cancer (excluding skin cancer) among women in the United States with more than 175,000 new cases anticipated in 1999. The requirement of estrogens for normal breast development is well documented (1). However, estrogens also have been linked to breast cancer, presumably through their ability to stimulate cell proliferation (2) and inhibition of estrogen action therefore has been a primary objective in the treatment, and more recently the prevention of, breast cancer. For many years, estrogen effects were thought to be mediated by a unique, high affinity intracellular receptor protein, the estrogen receptor (ER), that is a member of a superfamily of transcription factors (3,4). The basic mechanisms of ER activity have been ascertained. Hormone binding to ER results in receptor homodimerization and binding to specific enhancer DNA elements located in the promoter regions of target genes (5,6). This process, which is accompanied by increases in ER phosphorylation (7-12), enables "activated" receptors to regulate the transcription of hormone-responsive target genes and the resulting changes in mRNA and protein synthesis are ultimately responsible for alterations in cellular function. The structural features of the estrogen receptor (ERα) responsible for hormone binding, dimerization, DNA binding and transcriptional activation have been identified (3,13-16) and these studies have provided the basis of our understanding of the molecular mechanisms by which estrogens regulate the growth and differentiation of mammary tissues.

Clearly, the transcriptional activity of the ER is regulated by estrogens, such as 17β-estradiol (E_2). However, the ERα also can be activated in the absence of exogenous ligand by agents that stimulate intracellular signal transduction cascades (EGF, IGF-1, heregulin, dopamine, TPA and cAMP) (7,17-23) or inhibit protein phosphatases (okadaic acid) (19). Furthermore, cyclin D1, independent of cyclin-dependent kinases, also can activate the ER in the absence of estrogen (24). The ERα knock-out mouse model confirms that ERα is required for some but not all *in vivo* EGF effects and established the importance of ligand-independent activation of ER to physiological events (25). Most of these ligand-independent activation pathways (with the exception of cyclin D1) increase receptor phosphorylation (7,12,23,26,27) and mutation of the only known ligand-independent (EGF) phosphorylation site (serine^{118}) to an alanine residue abolishes EGF activation of the ER (28), suggesting that phosphorylation may play an important role in these activation pathways. However, this point mutant does not block cAMP-mediated gene expression and different domains are required to respond to EGF and cAMP signaling pathways (29), suggesting that multiple mechanisms must exist to enable ER to activate target gene expression in response to diverse regulatory events.

In 1996, a new member of the nuclear receptor superfamily was cloned from a prostate cDNA library (30). When the resulting cDNA was sequenced and expressed, it became apparent that a novel estrogen receptor had been identified. This new member of the nuclear receptor superfamily was named ERβ, and the original estrogen receptor was renamed ERα. The ERβ binds to estradiol with an affinity (K_d 0.4 nM) similar to ERα and binds to the same DNA response element as ERα (30-32). Thus, it is reasonable to predict that ERβ regulates the expression of <u>at least</u> a subset of ERα target genes. However, the relatively undeveloped mammary glands in the ERα knock-out mouse indicate that ERβ is not equivalent to ERα (33). The reasons for this are unclear, but could be related to differential expression and/or differences in the ability of α and β receptors to activate target gene expression. Mouse, rat and human ERβs are approximately 65 amino acids smaller than their corresponding α-receptors, and the

A/B, D and F regions (**Figure 1**) are poorly conserved (30,32,34). Furthermore, the ligand binding domains (region E) of ERα and ERβ are only ~55% identical and rat ERα and ERβ receptors do not bind equally well to all ligands (31). The expression patterns of ERα and ERβ mRNAs are different but overlapping (31) suggesting that the genes for ERα and ERβ are independently regulated. However, both ERα and ERβ mRNA have been detected in human mammary gland, breast tumors and several human breast cancer cell lines (35,36). Taken together, these data suggest that ERβ is likely to play a role in mediating estrogen action in mammary gland, but that this receptor is unlikely to be functionally equivalent to ERα.

The identification of a second estrogen receptor raised a number of important biological questions such as, what is the expression of ERβ, relative to ERα, in normal and malignant mammary tissue? However, knowledge regarding the expression of ERβ in mammary gland will be of limited value without detailed information on the transcriptional activity of ERβ. Do ERα and ERβ respond similarly to ligand-independent pathways? Are currently used antiestrogens equally effective antagonists of ERα and ERβ? Do ERα and ERβ activate the same target genes to a comparable extent? Studies of this nature will provide the information necessary to determine whether resources are required to develop new strategies to more effectively and/or selectively block estrogen and estrogen receptor (α and β) action. Indeed, if ERα and ERβ are not functionally equivalent, experiments, such as those reported below, will provide a solid foundation upon which new strategies to regulate ERα and ERβ biological activity can be developed. Moreover, comparing and contrasting the structure/function relationships of ERα and ERβ with respect to activation by ligand-independent pathways represents a novel approach to study mechanistic questions relating to activation of gene expression in the absence of estrogens.

Body

A new member of the nuclear receptor superfamily, ERβ, has been identified that binds to estrogens with high affinity, and binds to the same DNA response elements as the classical estrogen receptor, ERα. Both of these ligand-regulatable transcription factors possess a well-defined, centrally located, DNA binding domain and carboxy-terminal domain, which contains a ligand-dependent activation function (AF-2); however the amino terminus which possesses a second activation function (AF-1) is poorly conserved. Thus, it is highly likely that that the biological activity of ERβ will differ from that of ERα. This hypothesis is being tested in the following two technical objectives:

1. To determine if estrogen-independent signaling pathways can stimulate ERβ transcriptional activity.
2. To determine what regions of ERβ contribute to its estrogen-independent transcriptional activity and to compare these regions to known ERα activation functions to characterize the structural features of these receptors that contribute to their respective biological properties.

The first ERβ cDNA published was isolated from a rat cDNA library and encoded a protein of 485 amino acids (30). Based on this report other investigators cloned the cDNAs for human and mouse ERβ, which encoded proteins of 478 and 485 amino acids, respectively (32,34). In

order to begin our studies of the ability of cell signaling pathways to activate ERβ-dependent transcriptional activity, it was necessary to construct expression vectors for ERα and ERβ such that each cDNA would be expressed from the same vector backbone. This is important to ensure that any differences in gene expression observed in subsequent studies could be attributed to differences in the receptors themselves and not to alterations in the relative expression of these proteins. Our ERβ cDNA was obtained in the vector, $pCMV_5$. Therefore, the ERα cDNA was isolated from the pJ3Ω vector (37), and subcloned into the *Eco*RI site of $pCMV_5$. Upon accomplishing this task, the transcriptional activity of ERα and ERβ was characterized in a transient transfection system to assess their respective abilities to regulate synthetic target gene expression in an estrogen-dependent manner. In order to perform these experiments, expression vectors for either ERα or ERβ were cotransfected into HeLa cells in the presence of a synthetic target gene, ERE-E1b-Luc, which consists of an estrogen response element (ERE) upstream of the E1b TATA box and the luciferase gene. When 10 ng of the respective expression vectors were used, it was noted that in estrogen-treated cells expressing ERα that the transcriptional activity was approximately 5-fold higher than in cells expressing ERβ (**Figure 2**). However, in titration experiments, increasing the amount of ERβ expression vector resulted in higher luciferase gene expression, suggesting the possibility that the relatively low ERβ transcriptional activity was due to poor expression of ERβ protein from the vector. Because the original ERβ cDNA had a very long (423 nucleotides) 5'-untranslated region (UTR), the possibility that this region of the cDNA was affecting the relative expression of ERβ was examined. An ERβ expression vector was constructed in which the first 399 nucleotides were removed by restriction digestion with *Acc*I and subcloning back into $pCMV_5$, and the resulting $ER\beta_s$ expression vector was again tested in transient transfection assays. This construct produced a receptor that was more active than the original ERβ, such that ERα was only 2.9-fold more active than $ER\beta_s$ (**Figure 3**). As a control, the parent vector, $pCMV_5$, was also transfected into cells with the ERE-E1b-Luc reporter gene, and as expected, no significant activity was observed, indicating that target gene expression was ER-dependent. In a more detailed experiment, increasing levels of expression vector for the original ERβ expression vector (hereafter referred to as $ER\beta_L$) and the modified expression vector ($ER\beta_S$) were titrated into cells along with the target gene, and luciferase activity was assessed. This experiment confirmed that $ER\beta_S$ stimulated more gene expression when low levels of expression vector (*e.g.* 10 ng) were used (**Figure 4**). However, when higher levels of expression vector were employed, the $ER\beta_L$ construct produced more luciferase activity. This suggested that the putative 5'-UTR region might be controlling the expression of ERβ protein in HeLa cells, and that alterations in receptor levels were contributing to the relative differences obtained in the *trans*-activation assays.

In order to determine whether the relative differences in target gene expression observed in cells transfected with the two ERβ expression vectors reflected variations in the relative expression of $ER\beta_S$ and $ER\beta_L$ protein, both hormone binding and Western blot analyses were performed. Increasing amounts of expression vectors for $ER\beta_L$, $ER\beta_S$, or $pCMV_5$ were transfected into HeLa cells, and 24 hours thereafter, cell extracts were prepared. Binding assays were performed with [^{3}H]estradiol in the presence and absence of a 100-fold excess of unlabelled 17β-estradiol. Separation of free and bound steroid was achieved with hydroxyapatite (38). This analysis revealed that the $ER\beta_S$ form of receptor was expressed, on average, at approximately 1.4 times the level of the original ERβ construct (**Figure 5**), supporting the hypothesis that the

higher apparent transcriptional activity of $ER\beta_S$ may simply reflect its relatively higher level of expression. No binding was detected in cells transfected with the $pCMV_5$ parent vector alone. In order to confirm these observations, Western blot analysis was performed with extracts prepared from cells transfected with $pCMV_5$, the original $ER\beta_L$ or the truncated $ER\beta_S$ expression vectors. Several commercial manufacturers have developed commercially available antibodies against ERβ since submission of this proposal. We evaluated antibodies from Affinity Bioreagents and Upstate Biotechnology, and in pilot experiments found that the Affinity Bioreagents antibody was able to recognized ERβ with superior specificity and affinity. This antibody was used in the Western blot analysis presented here, and will be used in subsequent years to examine ERβ phosphorylation. The availability of this antibody has made it unnecessary to generate an expression vector for Flag-epitope-tagged ERβ at this time. As shown in **Figure 6**, the expression level of $ER\beta_S$ was approximately 10-fold greater than that achieved for the original clone ($ER\beta_L$). More importantly however, was the observation that the two forms of ERβ migrated with different mobilities. The original ERβ construct appeared to encode a protein of ~60 kiloDaltons, while the expression vector in which the putative 5'-UTR had been deleted encoded a protein of ~56 kiloDaltons. This prompted us to sequence the putative 5'-UTR and upon doing so, an additional nucleotide (a C residue at nucleotide position 324 relative to the originally published sequence, reference (30)) that had not been reported in the original publication or database deposit of the ERβ sequence was discovered. Inclusion of this additional nucleotide extended the open reading frame for ERβ by an additional 45 amino acids. Within the last year, several other laboratories also have reached the same conclusion about the size of the full-length form of ERβ (39,40). Therefore, there are two forms of ERβ that we and others have investigated to date. The full-length form is 530 amino acids in length, while the truncated version is 485 amino acids long. Although we plan to use the full-length form for all of our subsequently planned studies, some of our early ERβ functional analyses have been performed with expression vectors for the short form of ERβ.

We have begun our analyses of the ability of ERβ to be activated by ligand-independent signaling pathways. For these studies, we have compared the activity achieved in cells transfected with ERβ (both long and short forms) in comparison to experiments that were performed in parallel examining the activity of ERα. We began by examining the receptor activity in cells that were treated with forskolin and isobutylmethylxanthine (IBMX). Forskolin is an activator of adenylyl cyclase and IBMX is a phosphodiesterase inhibitor, and treatment of cells with these compounds results in an increase in intracellular cAMP production. In transient transfection assays, 10 μM forskolin and 100 μM IBMX stimulated the activity of ERβ by 2.4-fold while the activity of the short from of ERβ ($ER\beta_S$) was stimulated only 1.9-fold (**Figure 7**). In comparison, the activity of ERα was stimulated by 2.4-fold. Thus, intracellular cAMP signaling pathways have the potential to activate the transcriptional activity of ERα and both forms of ERβ. Because these receptors have regions of high (DNA and ligand binding domains) and low identity (amino-terminal domains), it will be possible in the upcoming year to examine the structural regions that enable both receptors to respond to this signaling pathway. Other signal transduction pathways outlined in the original proposal will be examined in year two [*e.g.* EGF, IGF, okadaic acid (an inhibitor of protein phosphatase 2A), phorbol-12-myristate-13-acetate (an activator of protein kinase C pathways) and cyclin D_1].

The ability of antiestrogens to block cAMP-dependent activation of ERβ-dependent transcription was also assessed. Both the mixed antiestrogen, 4-hydroxytamoxifen (4HT) and the pure antiestrogen, ICI 164,384, were able to inhibit activation by this ligand-independent activation pathway (**Figure 8**). This contrasts with the inability of 4HT to block cAMP-dependent activation of ERα transcriptional activity (41), and the structural differences between the two receptor isotypes will be examined in the coming year to understand the mechanistic basis for these contrasting responses to antiestrogen treatment.

With respect to the second technical objective, work in the first year of this project has focussed on constructing vectors that will enable us to examine the structural features that enable ERα and ERβ to respond in equal or dissimilar ways to various estrogen-independent signals. Expression vectors for chimeric proteins have been generated in which the amino-terminal domains of ERα and ERβ (individually), have been fused *C*-terminal to the heterologous Gal4 DNA binding domain (DBD). These constructs isolate the A/B region which encompasses the AF-1 domain from the remainder of the respective receptor and will facilitate an examination of their transcriptional activity in response to ligand-independent signaling pathways. To assess the transcriptional activity of the A/B regions of ERα and ERβ in unstimulated cells, the respective expression vectors were transiently transfected into HeLa cells along with a synthetic target gene consisting of 5 copies of the response element to which the Gal4 DBD binds. This experiment demonstrated that the A/B domain of ERα has significantly more transcriptional activity than the Gal4 DBD alone (**Figure 9**). In contrast, the A/B domain of ERβ has weak activity in comparison to ERα, although it is distinguishable from that observed for the GAL4 DBD alone. These expression vectors will be used in the following year to examine the ability of signaling pathways to alter the activity of this isolated activation function. Expression vectors for ERα and ERβ deletion mutants in which the A/B domains have been deleted, or in which their E/F regions (lacking the DNA binding domain) will be fused *C*-terminal to the Gal4 DBD are currently being prepared. Collectively, these constructs will enable us to compare and contrast the ability of various estrogen-independent signaling pathways to activate a) the full-length receptor, b) the A/B region (AF1), c) the DNA and ligand binding domain and d) the ligand binding domain alone (AF2).

Finally, we have generated chimeras of ERα and ERβ in which the A/B region of the ERα receptor was substituted for the A/B region of ERβ to create a ERαβ chimera and *vice versa*. These constructs have been tested for their ability to be activated by 17β-estradiol (**Figure 10**) and as expected, we have observed that the ERα receptor is more active than ERβ or either of the two chimeras (ERαβ or ERβα). Thus, we have prepared chimeras that will enable us to examine in the context of a full-length receptor whether estrogen-independent signals require the amino and carboxy-termini of the same receptor subtype, or whether the two receptor forms can substitute for one another.

Key Research Accomplishments

1. ERα is more active than $ER\beta_S$. Furthermore, $ER\beta_S$ is more active than $ER\beta_L$ in response to 17β-estradiol stimulation for low amounts of transfected expression plasmids, whereas $ER\beta_L$ is more active than $ER\beta_S$ when higher levels of expression plasmid are transfected into cells.

2. $ER\beta_S$ is expressed at a higher level than $ER\beta_L$ for equal amounts of transfected expression vectors as indicated by hormone binding (≤2-fold) and Western blot analyses (>10-fold).
3. Forskolin/IBMX activated ERα and $ER\beta_L$ approximately 2.4-fold whereas $ER\beta_S$ was activated even less than $ER\beta_L$.
4. The mixed antiestrogen, 4-hydroxytamoxifen, and the pure antiestrogen, ICI 164,384, inhibited the ability of ERβ to activate target gene expression in response to estrogen and cAMP signaling pathways.
5. Expression vectors for chimeras of the A/B regions of ERα and ERβ with the GAL4 DNA binding domain have been generated and characterized. The AF-1 activity of ERα is greater than the AF-1 activity of ERβ.
6. Expression vectors for chimeras of the *N*-termini and *C*-termini of ERα and ERβ have been constructed and characterized.

Reportable Outcomes

A portion of the work outlined in this progress report was presented at the 81st Annual Meeting of The Endocrine Society (San Diego, CA; June 12-15, 1999) in poster form. The abstract (see Appendix) was entitled "Activation of estrogen receptor-α and estrogen receptor-β by ligand-dependent and ligand-independent pathways".

Conclusions

The originally published amino acid sequence of ERβ represents an amino-terminally truncated form, which lacks the first 45 amino acids of this receptor isotype. In transient transfection assays, ERα is clearly more active than the long and short forms of ERβ. The potentially large differences in $ER\beta_S$ and $ER\beta_L$ expression levels indicate that their relative expression levels must be taken into account when considering transactivation activity. Furthermore, the AF-1 activity of ERα exceeds that of ERβ and this likely contributes to the relative differences in transcriptional activity observed for these two receptor isotypes.

Both isotypes of estrogen receptor (α and β) can be activated in the absence of exogenous estrogens. In cells treated with forskolin and IBMX, ERα and both forms of ERβ (short and long) were activated by a cAMP signaling pathway. This indicates that there is sufficient homology between these two receptor isotypes to mediate activation of gene expression by this signaling pathway. However, the responses of these receptors to inhibition of cAMP activation by 4-hydroxytamoxifen are distinct. This mixed antiestrogen inhibits cAMP-stimulated, ERβ-dependent transcriptional activity, while 4-hydroxytamoxifen further activates ERα stimulated by cAMP signaling pathways. Thus, there are differences between these two receptor isotypes with respect to their regulation by antiestrogens. Expression vectors for various ER deletion mutants have been constructed which will enable us in the upcoming year to begin to examine which region(s) of these receptors is responsible for the differences in response.

As anticipated, the experiments performed to date have provided information on the transcriptional activity of ERβ relative to ERα, as well as the ability of ERβ to respond to an alternative signaling pathway, induced by elevated intracellular cAMP, in the absence of estrogens. Using the expression vectors for mutant forms of ERα and ERβ constructed this year, information on the structural features of the two receptors that are critical for responsiveness to ligand-independent signaling pathways should be obtained. Taken together, this information will increase our understanding of the molecular mechanisms by which ERα and ERβ respond to cross-talk within a cell. It also will provide a framework for critical evaluation of whether it is possible to selectively regulate ERα and ERβ transcriptional activity.

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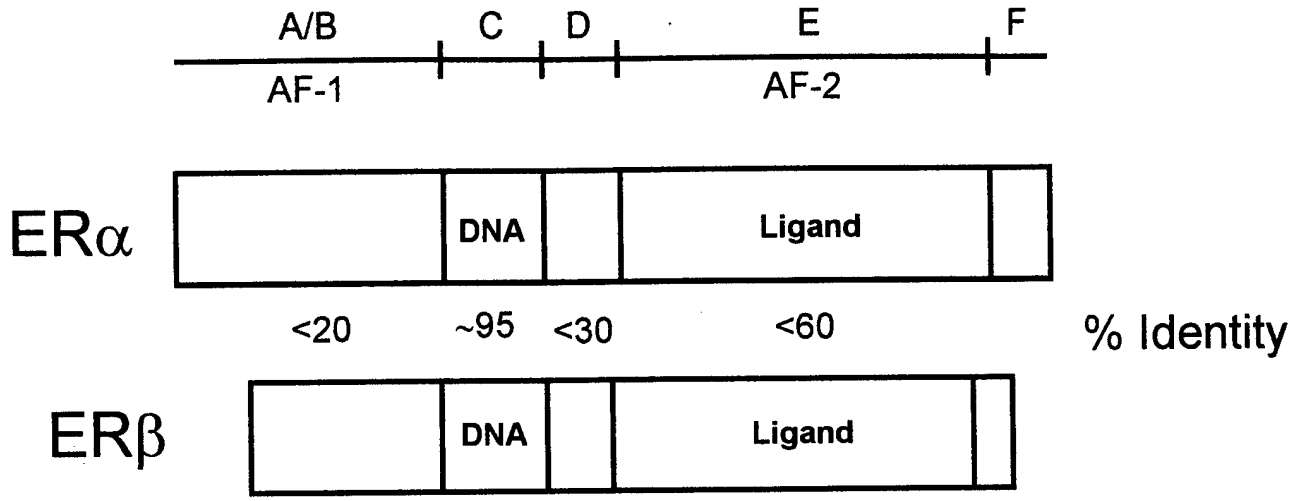

Figure 1: Structures of ERα and ERβ. The *top panel* represents the location of various regions of estrogen receptor (A to F) and its activation functions (AF-1 and AF-2). The bottom panel represents the comparative structure of ERα and ERβ. Values given between the two receptor forms represent approximate homologies in their respective amino acid sequences.

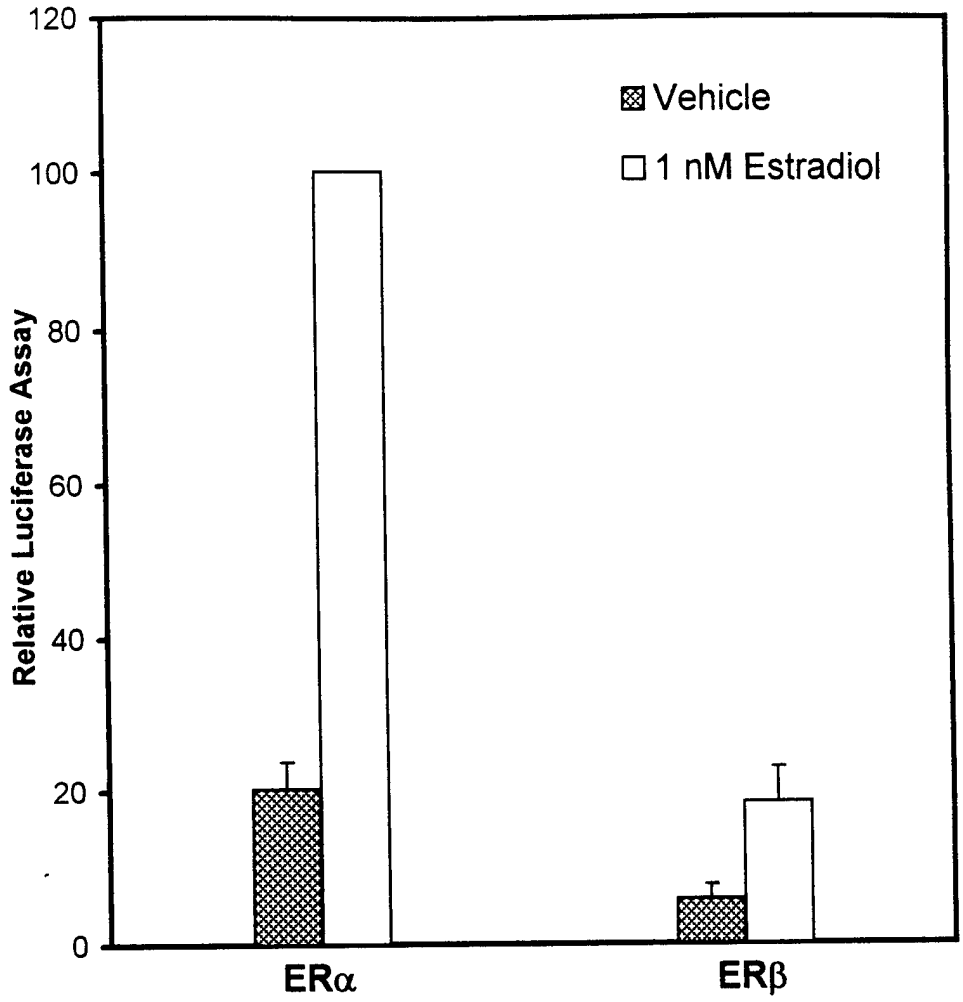

Figure 2: ERα is More Active than ERβ in Response to Stimulation with 17β-estradiol. HeLa cells were co-transfected with 1 μg ERE-E1b-Luc reporter plasmid and either 10 ng pCMV$_5$-ERα (ERα) or 10 ng pCMV$_5$-ERβ (ERβ). Luciferase measurements were corrected for protein values and standardized to ERα activity stimulated by 1 nM estradiol. Transfections were performed in duplicate and values represent the mean ± SEM of three experiments.

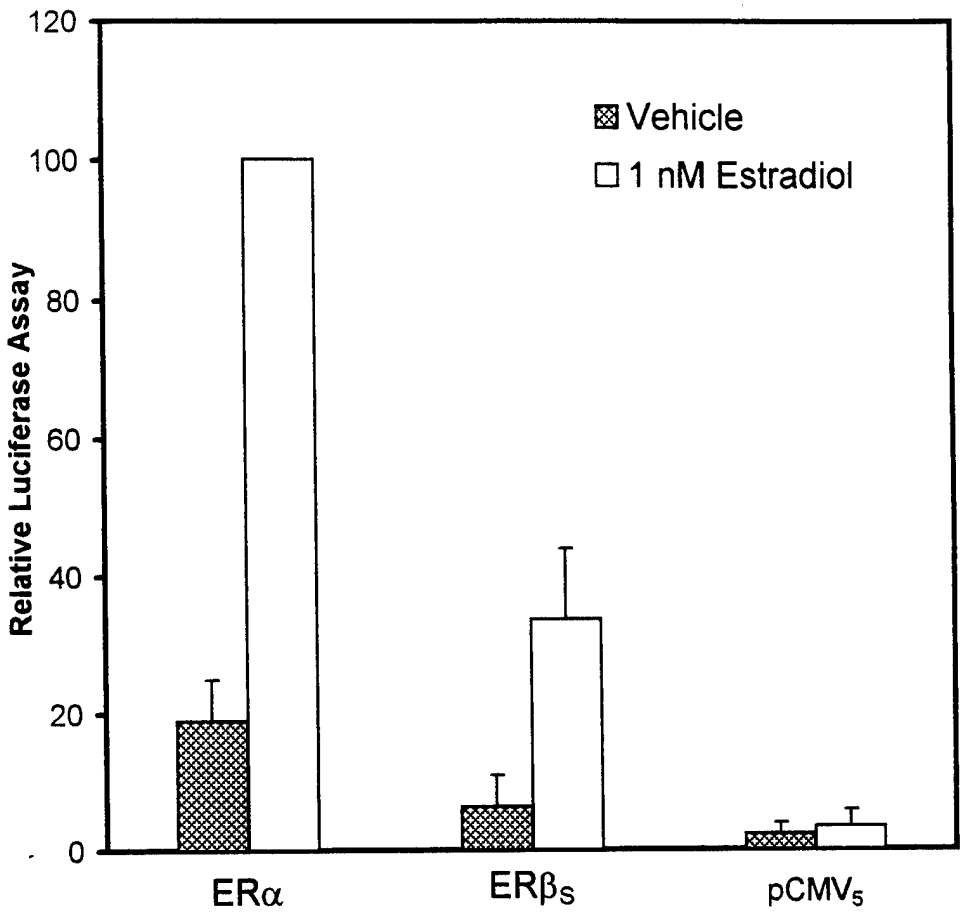

Figure 3: ERα is More Active than ERβ-short in Response to Stimulation with 17β-estradiol. HeLa cells were co-transfected with 1 μg ERE-E1b-Luc reporter plasmid and either 10 ng $pCMV_5$-ERα (ERα) or 10 ng $pCMV_5$-ERβ-short ($ERβ_S$). Luciferase measurements were corrected for protein values and standardized to ERα activity stimulated by 1 nM estradiol. Transfections were performed in duplicate and values represent the mean ± SEM of three experiments.

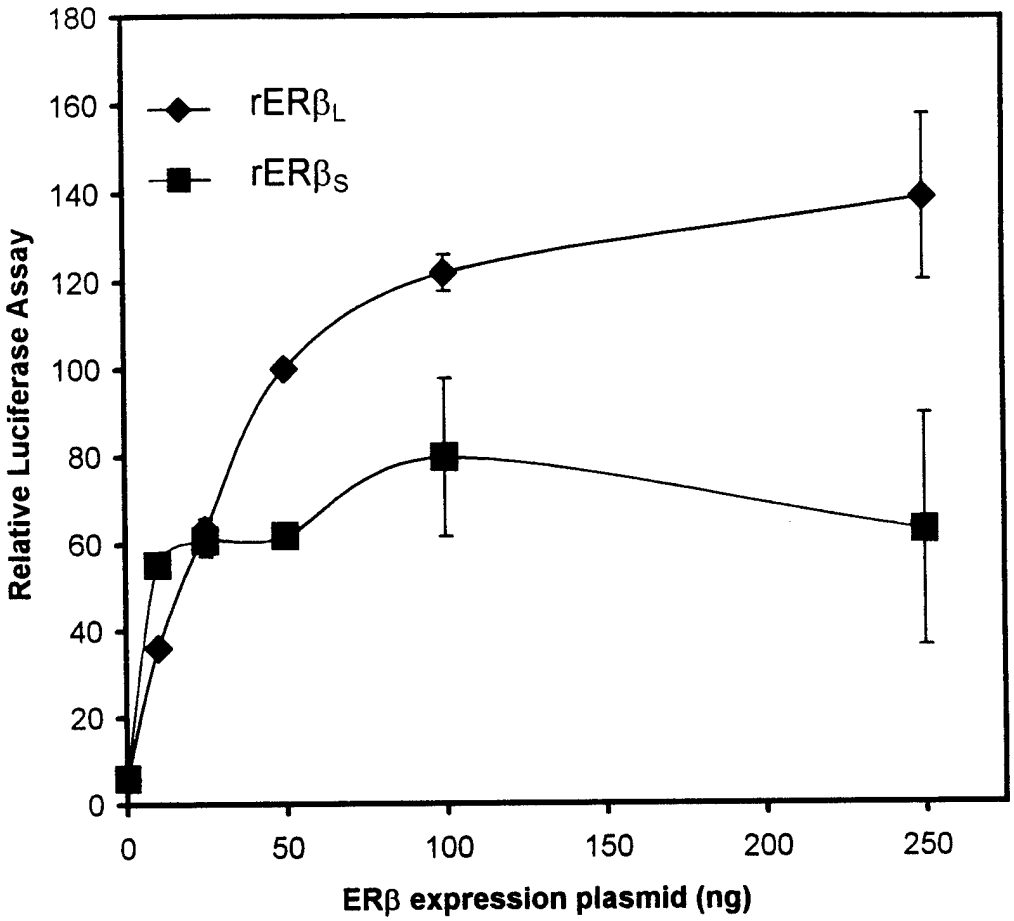

Figure 4: Comparison of ERβ-long and ERβ-short Transcriptional Activity. HeLa cells were transfected with 1 μg ERE-E1b-Luc, 100 ng CMVβgal, and the indicated amounts (*x-axis*) of $pCMV_5$-$ER\beta_L$ or $pCMV_5$-$ER\beta_S$ expression plasmids. Luciferase measurements, normalized to CMVβgal activity, are the results of stimulation with 1 nM estradiol. Transfections were performed in duplicate and values were standardized relative to values obtained for 50 ng of $ER\beta_L$. Values represent the mean ± SEM of four experiments.

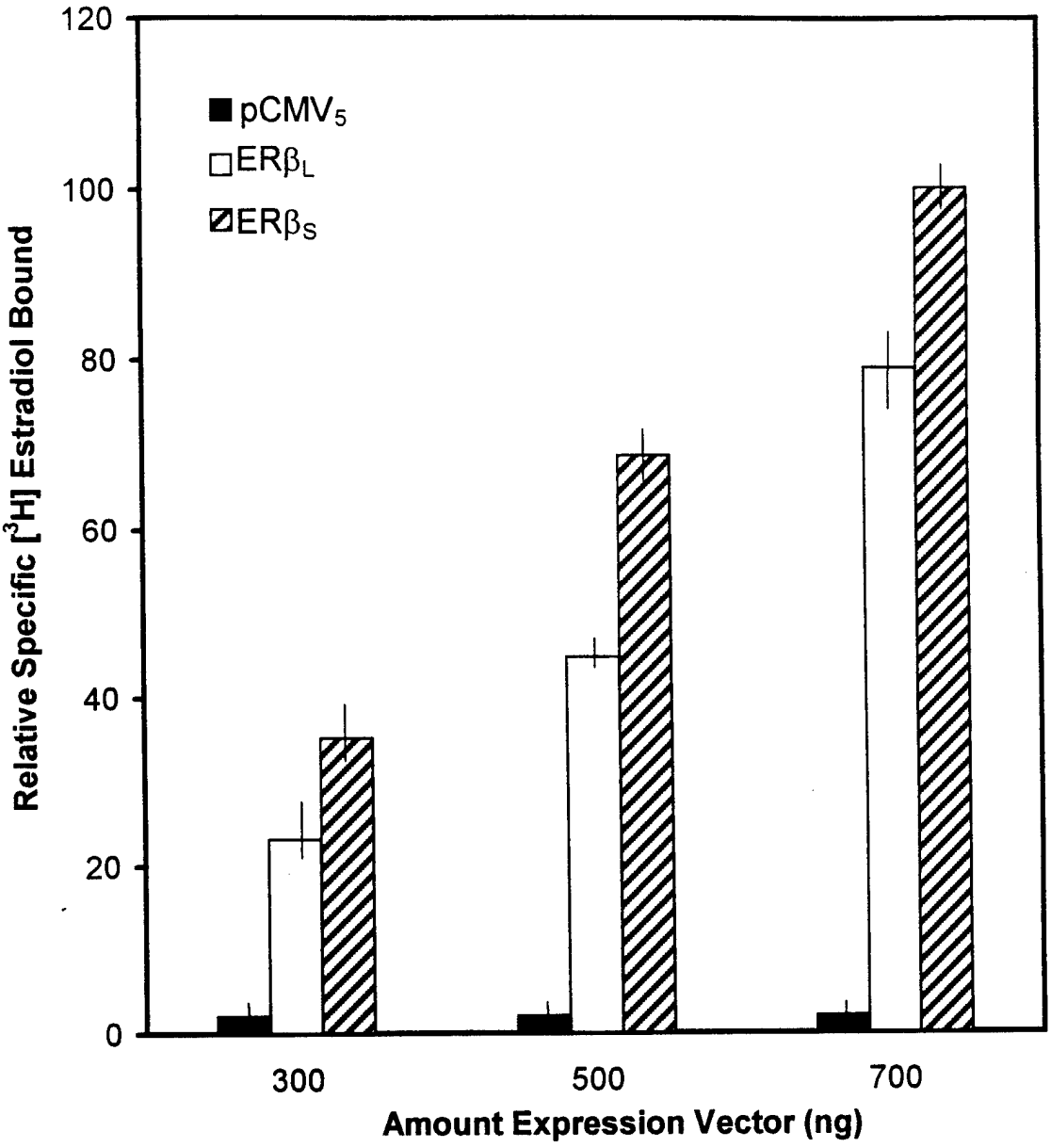

Figure 5: Relative Expression of ERβ-short and ERβ-long Determined by *In Vitro* Hormone Binding Analyses. HeLa cells were transfected with CMVβgal and the indicated amounts of either pCMV$_5$-ERβ_S or pCMV$_5$-ERβ_L expression plasmids, or the parent vector (pCMV$_5$). Binding assays were performed in duplicate and values represent the mean and range of relative specific counts bound, normalized to CMVβgal activities for two experiments.

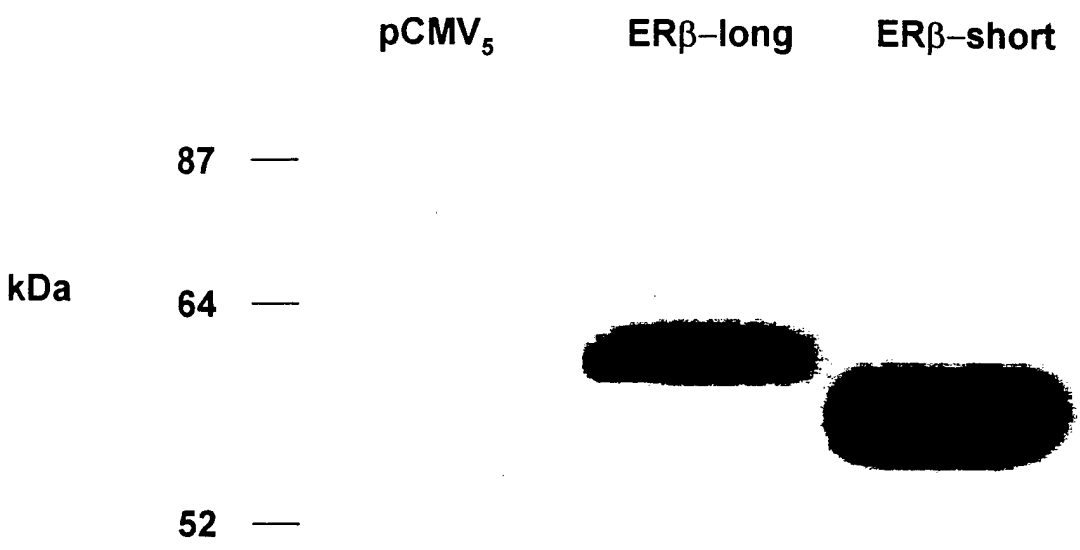

Figure 6: Relative Expression of ERβ-short and ERβ-long determined by Western Analyses. HeLa cells were transfected via adenovirus with $pCMV_5$-$ER\beta_S$, $pCMV_5$-$ER\beta_L$, or the parent vector and cells harvested twenty four hours after transfection. Cells were lysed in ER extraction buffer, protein concentrations determined by BioRad reagent, and an equal amount (25 μg) of protein was resolved by 7.5% SDS-PAGE. The resulting gel was blotted onto nitrocellulose membrane and an anti-ERβ antibody was employed for detection of the ER. Molecular size standards are shown to the *left* of the figure.

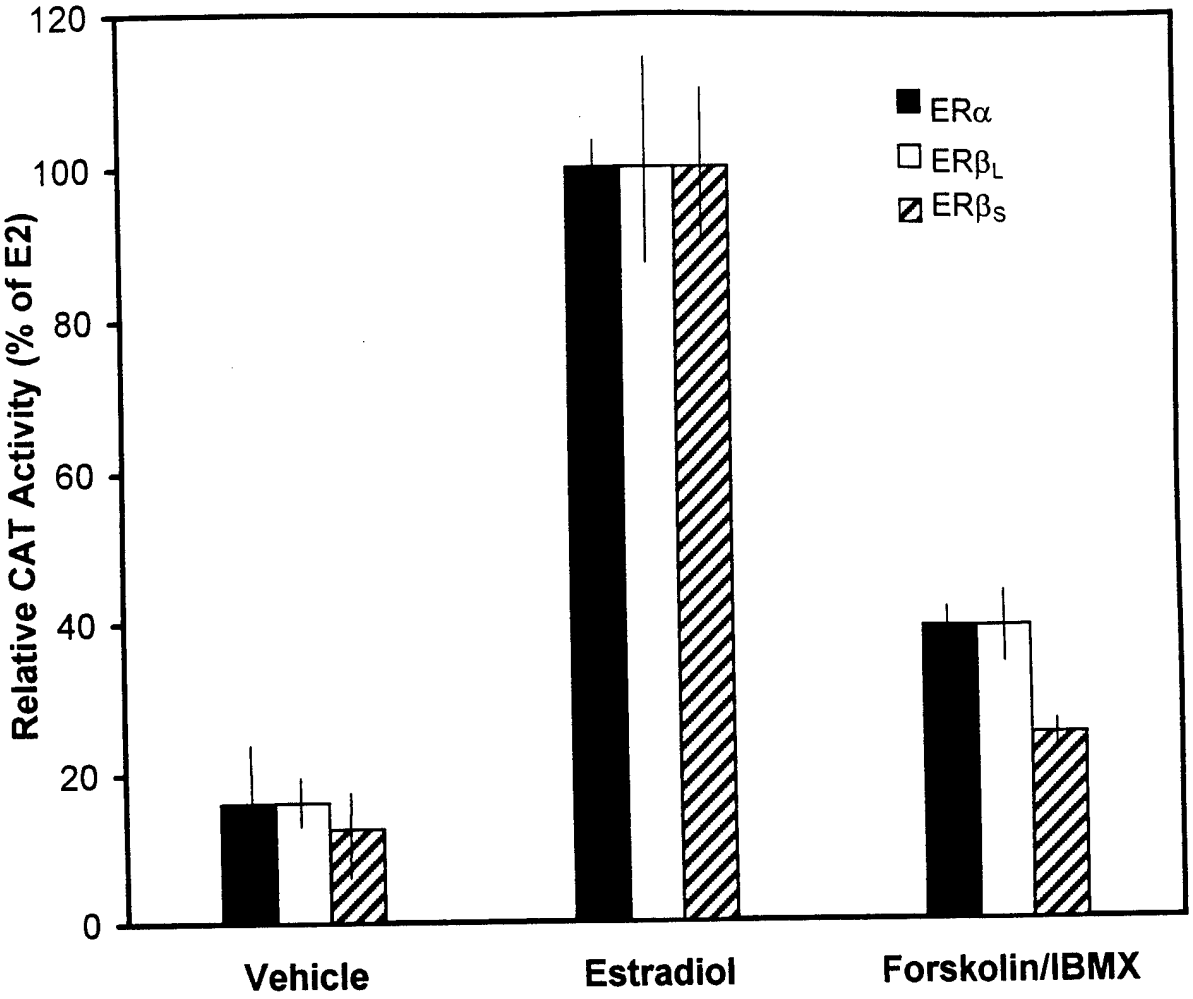

Figure 7: Forskolin/IBMX Activates ERα, ERβ-long and ERβ-short. HeLa cells were transfected with an ERE-E1b-CAT reporter, CMVβgal, and either pCMV$_5$-ERα, pCMV$_5$-ERβ$_L$ or pCMV$_5$-ERβ$_S$ expression plasmids and stimulated with EtOH, 1 nM estradiol, or 10μM forskolin + 100 μM IBMX. CAT measurements were made and corrected for CMVβgal activity, and values were standardized to the respective estradiol-stimulated activities. Transfections were performed in duplicate and values represent the mean and range for two experiments.

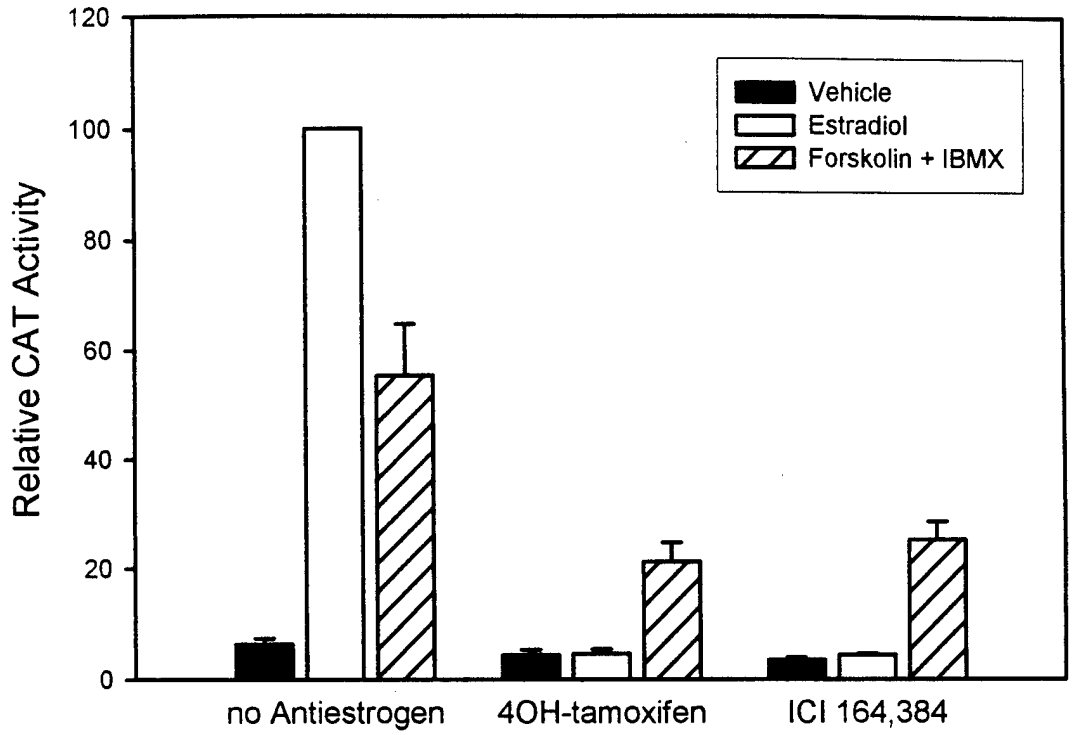

Figure 8: Activation of ERβ$_L$ Transcriptional Activity by Forskolin/IBMX and inhibition by Antiestrogens. HeLa cells were transfected with the 1 μg ERE-E1b-CAT and 250 ng of pCMV$_5$-ERβ$_L$ expression plasmid. Luciferase measurements, normalized to cellular protein, are the results of stimulation with 1 nM estradiol or 10 μM forskolin & 100 μM IBMX in the absence or presence of 100 nM 4-hydroxytamoxifen or 100 nM ICI 164,384. Transfections were performed in duplicate and values are standardized relative to values obtained for cells treated with estradiol alone. Values represent the mean ± SEM of five experiments.

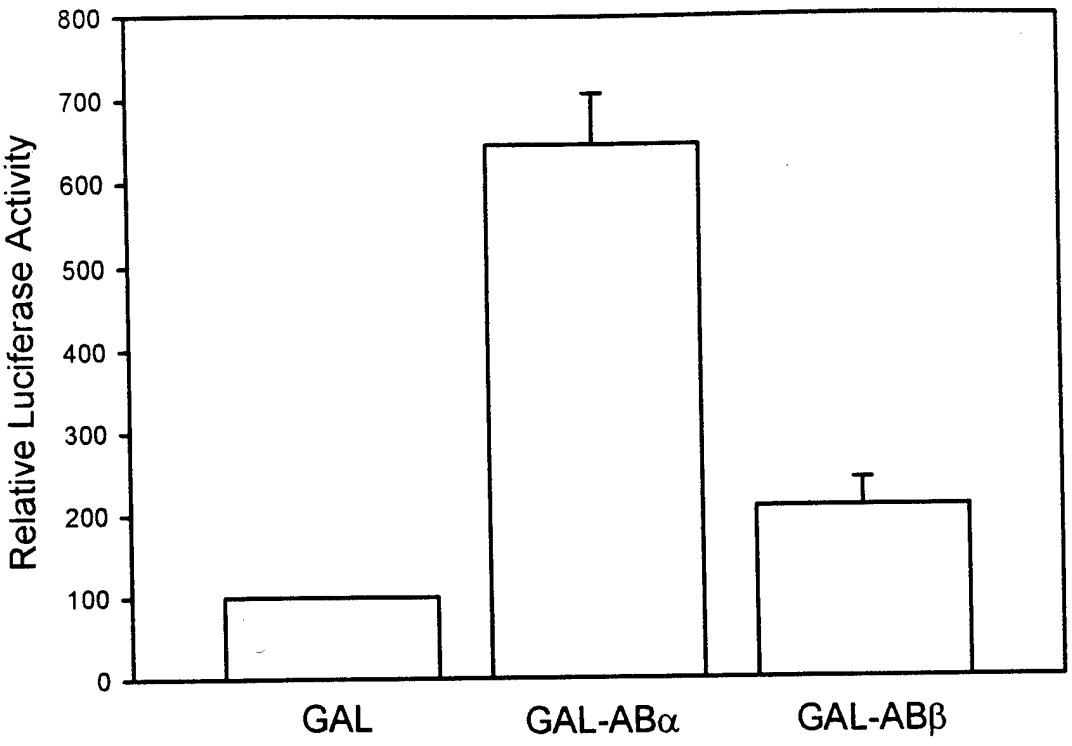

Figure 9: Transcriptional Activity of Gal-ABα and GAL-ABβ Chimeric Proteins. HeLa cells were transfected with 100 ng of expression vectors for the GAL4 DNA binding domain alone (GAL), or the GAL4 DNA binding domain fused to either the A/B region of ERα (GAL-ABα) or ERβ (GAL-ABβ) with 1 ug of pG5-Luc. Luciferase measurements were normalized to cellular protein. Transfections were performed in duplicate and values are standardized relative to values obtained for cells transfected with the expression vector for GAL4 DNA binding domain alone. Values represent the mean ± SEM of four experiments.

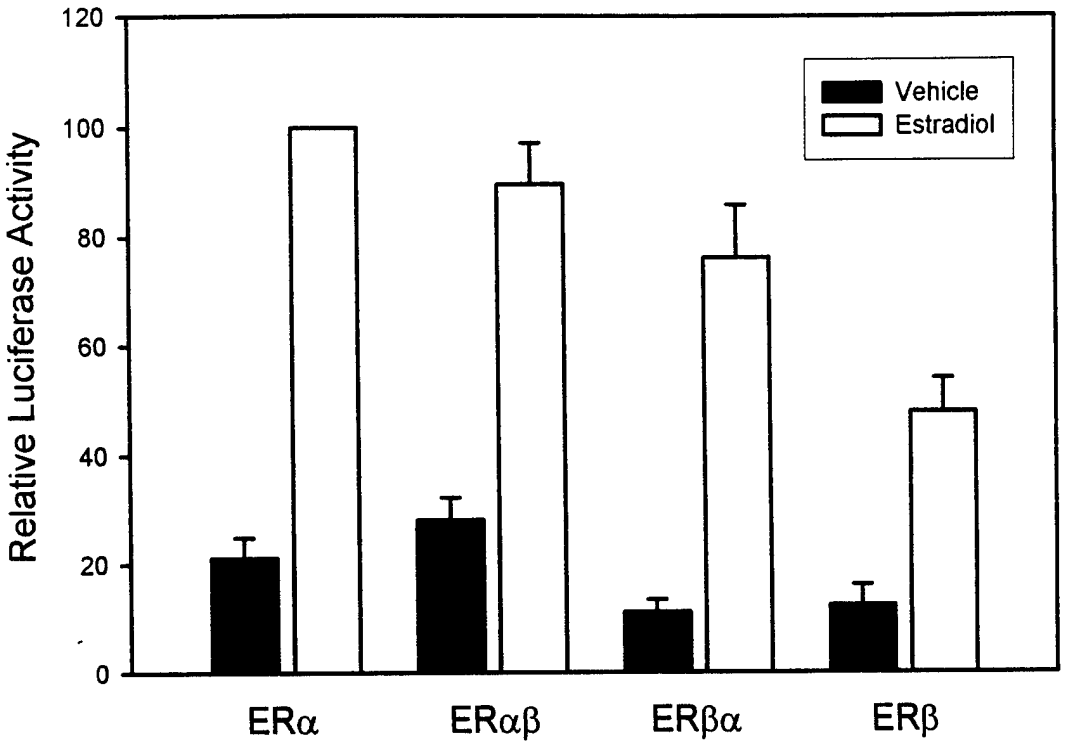

Figure 10: Transcriptional Activity of ERα and ERβ Chimeras. HeLa cells were transfected with 100 ng of expression vectors for ERα, ERβ or chimeras of the two receptors, ERαβ or ERβα with 1 ug of ERE-E1b-Luc. Luciferase measurements were normalized to cellular protein. Transfections were performed in duplicate and values are standardized relative to values obtained for cells transfected with the expression vector for ERα and treated with 1 nM estradiol. Values represent the mean ± SEM of three experiments.

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ACTIVATION OF ESTROGEN RECEPTOR-ALPHA AND ESTROGEN RECEPTOR-BETA BY LIGAND-DEPENDENT AND LIGAND-INDEPENDENT PATHWAYS.

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The biological effects of estrogens are mediated via two intracellular proteins called the estrogen receptor-α (ERα) and estrogen receptor-β (ERβ). In addition to the originally characterized short form of rat ERβ which consists of 485 amino acids ($ER\beta_S$), a longer form of ERβ has been described which also possesses an amino-terminal extension of 64 residues resulting in a protein with a predicted size of 549 amino acids ($ER\beta_L$). To analyze potential functional differences between ERα and the short and long forms of ERβ, the ability of these receptors to transactivate target genes in transient transfection experiments in response to ligand-dependent and ligand-independent activation pathways was assessed in HeLa cells. The short form of ERβ was created by deleting the first 399 nucleotides of the long form, rat ERβ cDNA, and all receptor cDNAs were expressed from the same mammalian expression vector, $pCMV_5$. Western blot analysis indicated that the $ER\beta_L$ cDNA encoded a protein of ~60 kDa, whereas the $ER\beta_S$ cDNA produced an ~56 kDa protein. Although both isoforms of ERβ were significantly less active than ERα in response to estradiol treatment, within the linear range of the assay $ER\beta_S$ stimulated gene expression up to 1.5-fold higher than $ER\beta_L$. However, when very high levels of $ER\beta_L$ and $ER\beta_S$ expression vectors were introduced into cells, $ER\beta_S$-stimulated gene expression was only 70% of that induced by $ER\beta_L$. Hormone binding assays and Western blot analyses were performed to determine if differences in $ER\beta_L$- and $ER\beta_S$-dependent reporter gene activity were due to alterations in the relative expression of the two receptor isoforms. Hormone binding assays indicated up to 2-fold higher expression for $ER\beta_S$ in comparison to $ER\beta_L$. Although this trend was confirmed by Western blots, this analysis indicated that when equal amounts of expression vector were introduced into HeLa cells, $ER\beta_S$ expression was >10-fold more abundant than $ER\beta_L$. Dopamine-initiated intracellular signaling pathways have been demonstrated to activate ERα, and the ability of the synthetic D1 subtype dopamine receptor agonist, SKF-82958, to activate the long and short forms of ERβ in a ligand-independent manner was assessed. Whereas SKF-82958 clearly activated ERα up to 3.8-fold in a dose-dependent manner, activation of either $ER\beta_S$ or $ER\beta_L$ was very weak (≤1.5 fold). Taken together, these results indicate that the relative expression of estrogen receptor isoforms must be taken into account when assessing their relative biological activity. Furthermore, ERα and ERβ transactivate reporter gene expression differently when stimulated in a ligand-dependent or ligand-independent manner and this supports the hypothesis that these receptors exert unique biological activities *in vivo*. Supported by a grant from the Department of Defense Breast Cancer Research Program (DAMD17-98-1-8282).